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14. ABSTRACT

BMP activity is tightly regulated at extracellular and intracellular levels of signaling as the dosage of BMPs can have widely varied effects depending on the time, tissue and domain in which they are expressed. However, **the regulation of BMP signaling within the MG, both during normal development and oncogenesis, remains largely unknown.** Twisted gastrulation (TWSG1) is a conserved extracellular modulator of BMPs. It can promote or inhibit BMP signaling depending on the constituents of the extracellular milieu. *Twsg1* is expressed in mammary glands from wild type (WT) mice at 3, 6 and 8 weeks of life, and is differentially expressed in breast cancer, yet its role in MG development and breast cancer pathogenesis is unknown. This study is designed to determine how TWSG1 regulates the invasion of normal MG tissue during ductal elongation and the behavior of breast cancer cells.

15. SUBJECT TERMS
BMP, Mammary gland development, ductal elongation, breast cancer

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Introduction

It has been shown that BMP expression is differentially regulated in breast cancer and understanding the role BMPs play in cancer is an active area of research (1, 2). Since many developmental programs are co-opted or misregulated in tumorigenic cells, it is critical that we understand the role these molecules play during development (3, 4). Twisted gastrulation, an extracellular BMP binding protein, can modulate BMP signaling in a variety of tissues (5-7). We therefore decided to use a $Twsg1^{-/-}$ mouse model to first investigate the role of BMP binding proteins in mammary gland (MG) development and secondly to understand their role in breast cancer. Our preliminary data identified abnormal postnatal mammary gland morphogenesis in $Twsg1^{-/-}$ mice. Our current data demonstrates a role for TWSG1 in the regulation apoptosis in breast cancer (BC).

Body

Specific Aim 1: To identify binding partners of TWSG1 during mammary gland development.

Task 1: Quantitative real-time PCR analysis of expression of *Twsg1*, Bmp ligands and their receptors. Given that there is a fully formed ductal tree at birth, suggesting that embryonic development occurs normally, we chose to focus on BMP signaling pathway components in the postnatal MG. Using RT-PCR we assessed ligands, receptors and other BMP binding proteins to determine if members of a complete BMP pathway were being expressed and if so, at what developmental time point. We were able to detect multiple ligands, receptors and other BMP binding proteins at most time points (Figure 1). Using the RT-PCR information and the fact that the observed defects were present at the onset of puberty, roughly 6 weeks of age, but not at later time points we began our Q-PCR analysis at this time point. Analyzing pubertal duct cDNA we measured a significant reduction in downstream targets such as *Id-2, Msx1, Msx2* and *Gata-3* in the $Twsg1^{-/-}$ MG *(Appendix 1).*

Task 2: Localization of *Twsg1* expression by immunohistochemistry and *in situ* hybridization. A mouse carrying a LacZ reported in the *Twsg1* locus became available and we decided to use this approach rather than IHC or in situ hybridization. Upon whole mount analysis, we were able to demonstrate that
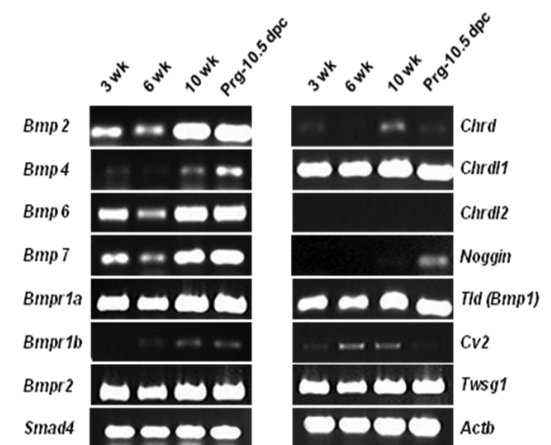
Figure 1. RT-PCR showing temporal expression of BMP pathway members in MGs at different postnatal developmental time points. *Actb,* beta-actin*, Chrd*, chordin; *Chrdl1*, chordin-like 1; *Chrdl2,* chordin-like 2; *Cv2,* crossveinless 2.
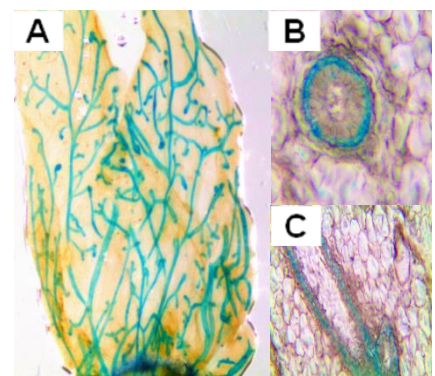
Figure 2. LacZ staining of whole mount (A) and sectioned (B,C) mammary glands isolated from heterozygous 2-month old virgin female carrying *LacZ* reporter gene in the *Twsg1* locus. (A) *Twsg1* is present in ductal structures (B,C) *Twsg1* expression is restricted to the myoepithelium (B,C).

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Twsg1 is expressed in the ducts of the pubertal MG. Sectioning of the MG showed that *Twsg1* expression is restricted to the myoepithelium (Figure 2).

Specific Aim 2: Characterize the ductal elongation defect in Twsg1-/- female mice.
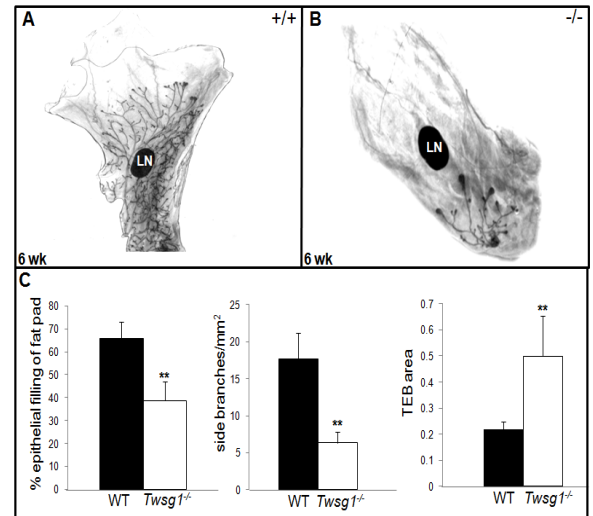
Task 1: Examine MG morphology at various stages of development. MG from 6 week old animals were harvested and whole mounts were examined for fat pad colonization, a measure of ductal elongation, branching and terminal end bud (TEB) size. In *Twsg1$^{-/-}$* the ductal tree colonized only 38% of the available fat pad while the WT tree had colonized 64% of the available fat pad. Furthermore, branching was significantly decreased in the *Twsg1$^{-/-}$* MG and the TEBs were significantly increased in size (Figure 3).

Figure 3. Loss of TWSG1 results in a delay of ductal elongation and a reduction in secondary branching. (A) MG from 6 week old wild type (WT) female mouse. (B) MG from 6 week old *Twsg1$^{-/-}$* virgin female mouse. (C) Fat pad colonization is significantly (p = 0.0032) delayed in *Twsg1$^{-/-}$* MGs at 6 weeks. Secondary branching is reduced in *Twsg1$^{-/-}$* MGs at 10 weeks (p = 0.005). TEBs are significantly larger in size in the *Twsg1$^{-/-}$* MGs at 6 weeks LN, lymph node.

Task 2: Examine changes in expression of downstream targets of BMP signaling in Twsg1-/- MG. In situ hybridization did not provide sufficient signal to determine changes in BMP downstream targets. Instead, QPCR was used to determine changes in downstream targets using K14 and GAPDH to normalize so we could identify changes occurring in the epithelium rather than the MG as a whole. Canonical targets were unchanged but a significant decrease in *Gata3* expression was identified (*Appendix 1*). Normal mouse mammary epithelium cells were treated with BMP and a significant induction of *Gata3* expression was observed.

Task 3: Determine the effect of TWSG1 deficiency on BMP signaling in MG. Whole glands were collected from WT and *Twsg1$^{-/-}$* at 6 weeks. Immunofluorescene and western blot was performed probing for pSMAD 1/5/8 which are the signal transducers of BMP. pSMAD was significantly reduced within the ducts of the *Twsg1$^{-/-}$* MG (Figure 4). It has been shown in other systems that TWSG1 can agonize BMP signaling (8, 9) however this is the first time that it has been demonstrated in mammals.

Task 4: Examine proliferation and apoptosis within MG. MGs from WT and *Twsg1$^{-/-}$* 6 week old animals were assessed by TUNEL for apoptosis and BrdU incorporation for proliferation. There was no significant difference in proliferation between WT and
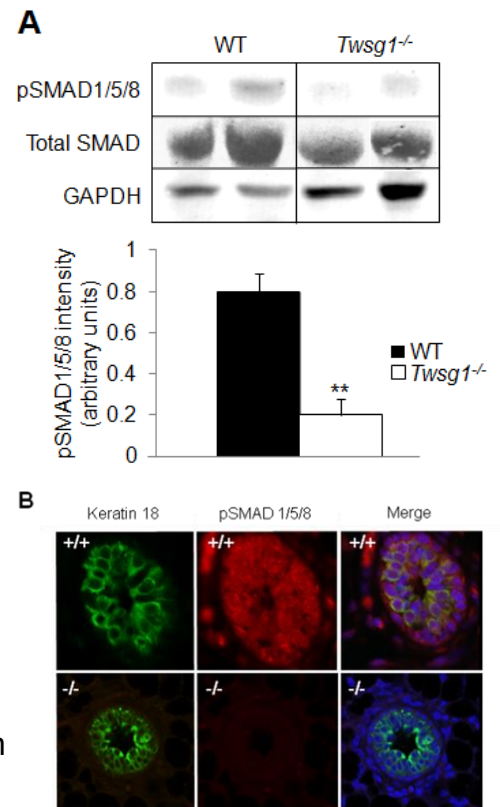
Twsg1^-/- however apoptosis was significantly reduced in *Twsg1^-/-* MGs. Further analysis also showed that there were significantly more epithelial cells in the *Twsg1^-/-* MG (*Appendix 1*).

Task 5. Examine if the ductal elongation defect is epithelial cell-autonomous. To determine if the observed defects were intrinsic to the MG rather than secondary to other factors, for example alterations in hormonal milieu, we performed transplantation experiments. Briefly, we removed the endogenous epithelium from 3 week old WT female mice and transplanted *Twsg1^-/-* or WT epithelium into the cleared fat pads. Mice were sacrificed 3 weeks later and there was either a significant delay (n=2) or no measurable outgrowth (n=3) of *Twsg1^-/-* MG tissue when compared to contralaterally transplanted WT tissue (data not shown). Along with elongation delay *Twsg1^-/-* transplanted epithelium appeared hyperplastic with occluded lumens when compared to WT (Fig. 3C). We also transplanted WT tissue in to *Twsg1^-/-* MGs cleared of endogenous epithelium and outgrowth was similar to that of the WT tissue transplanted into WT fat pad (*Appendix 1*).

Our preliminary data demonstrated overexpression of TWSG1 in BC by microarray (data not shown.) However, there was no clear correlation between the molecular subtype and TWSG1 expression in the patient population sampled. Immunohistochemistry demonstrated that TWSG1 was expressed in what appeared to be the myoepithelium of the normal breast and was expressed in a wide variety of BCs (Figure 5).

Figure 4. BMP signaling is reduced in MGs from *Twsg1^-/-* animals. (A) Western blot, showing two representative samples per group, detecting the levels of total SMAD, pSMAD 1/5/8 and GAPDH in MG lysates from WT and *Twsg1^-/-* 6 week old virgins. Total SMAD was normalized to GAPDH and a ratio of SMAD to pSMAD1/5/8 was calculated; $p \leq 0.002$. **(B)** Immunofluorescence staining for epithelial marker keratin 18 (green) and pSMAD1/5/8 (red). pSMAD1/5/8 is absent in *Twsg1^-/-* MGs

Specific Aim 3: To examine if TWSG1 is involved in breast cancer pathogenesis.

Task 1: Examine the expression of *Twsg1, Bmp2, Bmp4, Bmp7, Bmprla* and *Bmprlb, Bmprll* in ER positive/PR positive and triple negative breast cancer cell lines. We were able to determine that in all breast cancer cells (BCC) lines examined (MCF10A, SKBR3, HCC1569, MD-MBA-231, T47D and MCF7) transcripts for a complete BMP pathway were present and that *TWSG1* was expressed in all. When transcripts were quantified by QPCR, MD-MBA-231 and T47D expressed significantly higher levels of *TWSG1* while all others tested expressed the same levels as MCF10A (data not shown.) We chose to move forward with the MD-MBA-231 and select other triple negative breast cancer cells (TNBCC) in which to study the role of TWSG1.
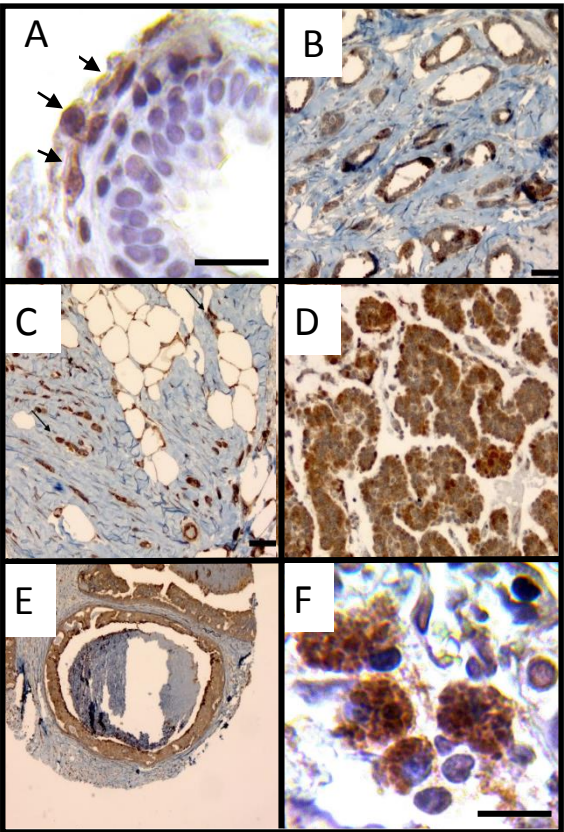
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Interestingly, we observed by RTPCR a pattern of ligand transcript switching from BMP2 to BMP4 and a switch from the expression of Chordin (CHRD) to that of Chordinlike 1 (CHRDL1) both are extracellular BMP binding proteins that can bind BMP and TWSG1 (Figure 6). To further confirm ligand transcript change we completed QPCR in MCF10As and MD-MBA-231s. MCF10As express BMP2 transcripts while MD-MBA-231s do but at a very low level and instead express BMP4 transcripts at a high level while MCF10As do not. The TNBCC line HCC 38 expressed low levels of TWSG1 and expressed both BMP2 and BMP4.

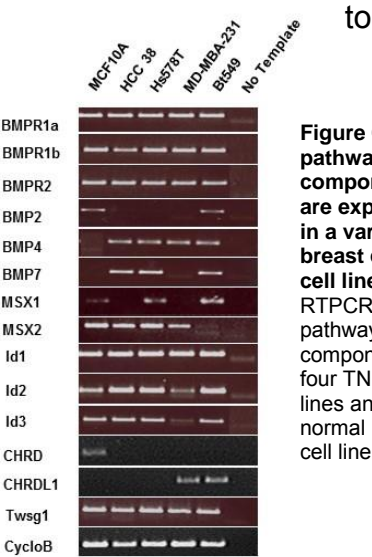**Task 2: Examine if overexpression or silencing of Twsg1 in breast cancer cell lines affects BMP signaling level.**

Cells were transduced with adenovirus carrying either a CMV-EGFP cassette or a double cassette of CMV-EGFP, CMV-hTWSG1.Transduced cells were plated onto chamber slides and stained with anti-pSMAD1/5/8. There were significantly more cells in the TWSG1 transduced cells with pSMAD localized to the nucleus (data not shown.) It was also observed that that there was an increase in cell size and an increase in the cytoplasmic to nuclear ratio. Western blotting showed a significant increase in pSMAD in TWSG1 overexpressing cells when compared to controls.

Figure 5. TWSG1 is expressed in a variety of breast cancers. A.) TWSG1 seems to be restricted to the myoepithelium. Expression is detected in B.) invasive ductal carcinoma C.) invasive lobular carcinoma D.) invasive papillary carcinoma E.) ductal carcinoma in situ F.) papillary carcinoma

Task 3: Determine if altered BMP level affects migration of breast cancer cells.

A transwell migration assay was used to determine if altering the levels of TWSG1 expression could change the migratory behavior of normal breast and BCCs. Cells were serum starved and then placed into migration chambers with 2% serum as a chemoattractant. The normal cell line, MCF10A, overexpressing TWSG1 was able to migrate significantly better than control tranduced. BCCs overexpressing TWSG1 also invaded better than control. This advantage could be inhibited by the blocking of BMP signaling by dorsomorphin, a BMP specific

Figure 6. BMP pathway components are expressed in a variety of breast cancer cell lines. A.) RTPCR of BMP pathway components in four TNBCCs lines and normal breast cell line
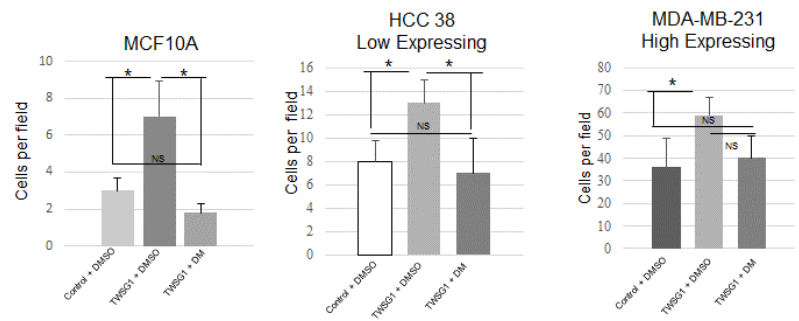
inhibitor (Figure 7). It has been established that BMPs can induce matrix metalloproteinase (MMPs) expression (10). Zymography demonstrated that overexpressing cells had more active MMP activity than control (Figure 8).

Given BMPs role in regulating apoptosis we investigated apoptosis in control and TWSG1 transduced cells by Apoptox assay and FLOW using Annexin V staining. TWSG1 overexpression in normal, MCF10A, cells led to an increase in apoptosis while overexpression in the TNBCC line MD-MBA-231 led to a reduction in apoptosis (data not shown.) There was no significant change in proliferation.

Figure 7. Invasion is increased as a result of TWSG1 overexpression. Transwell invasion assays show an increase in invasion in TWSG1 overexpressing cells. When cells are treated with dorsomorphin, invasion returns to baseline.

Figure 8. Zymography showing MMP 9 and MMP 2 activity increases in TWSG1 overexpressing cells.
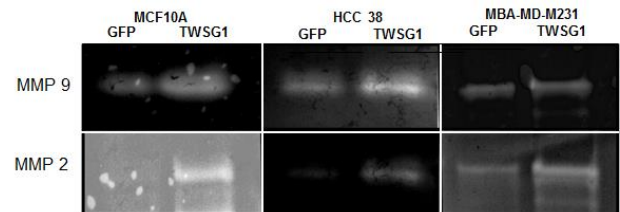
Task 4: Test if altered BMP level can induce cellular stress and activate p53 pathway. Given the variation in p53 for the cell lines chosen, this task was not pursued at this time. However, in future studies involvement of p53 and cellular stress will be assessed in normal cells.

Key Research Accomplishments
- **TWSG1 promotes BMP signaling in the developing mammary gland.**
- **TWSG1 null MGs have defects in ductal elongation, secondary branching and apoptosis.**
- **TWSG1 null MGs have occluded lumens and enlarged terminal end buds**
- **TWSG1 null ducts fail to restrict myoepithelium to the basal layer**
- **GATA-3, which is required for luminal identity, is reduced in TWSG1 null MGs**
- **Breast cancer cells express *Twsg1* and upregulate BMP 2.**
- **TWSG1 promotes BMP signaling in normal and breast cancer cells.**
- **TWSG1 promotes migration in normal breast cells.**
- **TWSG1 promotes apoptosis in normal breast cells.**
- **TWSG1 promotes invasion in a BMP dependent manner**
- **TWSG1 overexpressing cells have increased MMP activity.**

Mentorship and Development

I have attended Dr. Kaylee Schwertfeger's weekly lab meetings and have met with Dr. Douglas Yee once a semester to discuss findings. I have attended breast cancer journal club and have presented at these meetings. I attended the Mammary Gland Gordon Conference and the Society of Developmental Biologists' Annual Meeting where I presented a poster of my work and received critical feedback. The Gordon Conference allowed me to discuss my findings one on one with leaders in the field which dramtically enhanced my understanding and refined my work significantly. I also attended the breast cancer tumor board at Fairview University Hospital. These allowed me to connect my work to the real world of diagnosis and treatment of breast cancer. At the time of this submission I have secured a postdoctoral position in a breast cancer lab focused on tumor microenvironment.

Reportable Outcomes

Publications:

Forsman C, Ng B, Heinze R, Schmidt B. Sergi C, Gopalakrishnan R, Davydova J Yee D, Schwertfeger K, Petryk A. Twisted gastrulation overexpession promotes cell migration and invasion in breast cancer cells. In preparation.

Forsman CL, Ng BC, Heinze RK, Kuo C, Sergi C, Gopalakrishnan R, Yee D, Graf D, Schwertfeger KL, Petryk A. BMP-binding protein twisted gastrulation is required in mammary gland epithelium for normal ductal elongation and myoepithelial compartmentalization. Dev Biol. 2013 Jan 1;373(1):95-106

Abstracts

Forsman C, Ng B, Heinze R, Kuo C, Sergi C, Gopalakrishnan R, Yee D, Graf D, Schwertfeger K, Petryk A. Twisted Gastrulation regulates BMP signaling in the postnatal MG. Abstract to be presented at Gordon Research Conference on Mammary Gland Biology, Barga, Italy, June 2012.

Forsman C, Ng B, Heinze R, Kuo C, Sergi C, Gopalakrishnan R, Yee D, Graf D, Schwertfeger K, Petryk A. Twisted Gastrulation regulates BMP signaling in the postnatal MG. Abstract to be presented at Society for Developmental Biology Conference. Montreal, Canada. July 2012

Conclusion

This award has supported work that has identified a role for BMP signaling in the postnatal MG. Furthermore, our work has identified TWSG1 as an agonist of BMP signaling for the first time in a mammalian system. BMP signaling is often misregulated in cancer and understanding the role it plays during development will help inform how it might play a role in tumorigenesis. To investigate TWSG1 role in BC further we stained for TWSG1 in breast cancer tissue and have shown that TWSG1 is expressed in this tissue as well as the normal breast. We were able to show that a complete BMP pathway is transcribed in these cells. Curiously, we identified a change in ligand transcripts between normal and BCC as well as a difference in extracellular partners. We have shown an increase in apoptosis in normal overexpressing cells and a reduction in apoptosis in MD-MBA-231 overexpressing cells. In another TNBCC line that has low levels of TWSG1 we saw an increase in apoptosis when TWSG1 was overexpressed. There was no significant change in proliferation. Overexpressing cells migrated and invaded better than controls. Invasion advantage could be inhibited by blocking BMP signaling. We also saw and increase in MMP activity in overexpressing cells. Altogether, these data are beginning to highlight the importance of BMP signaling in regulating apoptosis in BC.

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APPENDIX 1

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Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor 3 (TGF3) superfamily, are morphogens that play diverse roles during embryonic development and postnatal life (Wagner et al., 2010). BMPs have been implicated in regulating tissue homeostasis in physiological states as well as pathological ones, such as cancer, by mediating basic biological processes including cell proliferation, migration, differentiation, apoptosis, and epithelial-stromal interactions (Buijs et al., 2012; Singh and Morris, 2010; Virtanen et al., 2011; Wang et al., 2011a). There is growing evidence that developmental programs, which are important during embryonic development and adult homeostasis, can be inappropriately reactivated during the initiation and progression of cancer (Bowman and Nusse, 2011; McEvoy et al., 2011; Wang et al., 2011b). Specifically, BMPs play important roles during embryonic mammary gland (MG) development and have been shown to be dysregulated in breast and other cancers often acting as a tumor suppressor or tumor promoter depending on dosage, time and tissue of expression (Bailey et al., 2007; Beppu et al., 2008; Cho et al., 2006; Hens et al., 2007; Liu et al., 2008). Given that developmental programs, such as BMP mediated processes, are co-opted or exploited by tumorigenic cells, it is important to understand what roles BMPs play during normal MG development and how their activity is regulated.

In both humans and mice, the MG is a dynamic organ that responds and develops over the lifetime of the female. Embryonic development establishes the rudimentary ductal tree that during puberty, pregnancy and lactation undergoes complex morphological changes (Hens and Wysolmerski, 2005; Hovey and Trott, 2004). In the mouse, MG development begins around embryonic day 10.5 (E10.5) when epithelial thickenings form placodes, which further thicken and invaginate as they respond to inductive signals from the underlying mesenchyme at around E13.5. At approximately E16.5, the primary bud epithelium proliferates and elongates into the developing fat pad forming a rudimentary tree. Development is arrested at this point until puberty. At puberty, terminal end buds (TEBs) form at the leading edge of the duct and are the site of active proliferation and apoptosis (Dulbecco et al., 1982; Humphreys et al., 1996). The TEB consists of bipotent cap cells that generate both cytokeratin 18 (K18) positive luminal and K14 positive myoepithelial cells. The latter remain in contact with the extracellular matrix (ECM) while the luminal cells polarize with their apical aspect oriented towards the lumen. In the TEB, proliferation creates excess body cells, which generate the mass of the duct as it elongates and a subset these cells undergo apoptosis to form the lumen. Clearing of excess cells is complete and the mature ductal structure can be seen beginning at the shoulder of the TEB (Humphreys et al., 1996). A mature duct consists of a layer, one to two cells wide, of K18 positive luminal cells and a single cell layer of K14 positive myoepithelial basal cells. Once the ducts reach the edges of the fat pad, TEBs are reabsorbed and elongation ceases. Concomitant with elongation, the ductal tree is further elaborated by a tightly regulated process of secondary and tertiary branching.

The dynamic changes seen in the MG are regulated by morphogens (Jardé and Dale, 2012; Schwertfeger, 2009), cytokines/growth factors (McNally and Martin, 2011; Watson et al., 2011) and hormones (McNally and Martin, 2011). Integration and regulation of these signals is required for the proper establishment of the MG anlagen and subsequent development through puberty and pregnancy. Past studies have suggested that BMP 2 and 4 play a role during MG development, including bud formation, epithelial mesenchymal communication, proliferation and lumen formation

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(Hens et al., 2007; Montesano, 2007; Phippard et al., 1996; van Genderen et al., 1994), but understanding of the regulation of BMP signaling during MG development is limited.

The BMP specific intracellular signal transducers of BMPs are SMAD1/5/8 proteins, which become phosphorylated upon binding of BMPs (BMP 2, 4, 6 or 7 among others) to their receptors (BMPR1A, BMPR1B and BMPR2). This binding can be facilitated by crossveinless 2 (CV2) (Zhang et al., 2010) or other receptor complex proteins. Once phosphorylated pSMAD1/5/8 binds SMAD4 and translocates to the nucleus where the complex acts as a transcription factor (Massague et al., 2005; Miyazono et al., 2005). In the extracellular space, availability of BMPs for binding to their receptors is regulated by the secreted proteins Chordin (CHRD), Chordin-like 1 (CHRDL1), Chordin-like 2 (CHRDL2), Noggin (NOG) and TWSG1 among others (Balemans and Van Hul, 2002). While CHRD and NOG are antagonists of BMP action, TWSG1 has a dual role, acting as either a BMP antagonist or as an agonist (Oelgeschlager et al., 2000; Ross et al., 2001) which has been shown in Danio rerio, Drosophila and Xenopus (Little and Mullins, 2004; Oelgeschlager et al., 2000; Shimmi et al., 2005). CHRD can be cleaved by the matrix metalloproteinase (MMP) BMP1, also known as tolloid, which can release ligand into the extracellular space. Cleavage of Chordin by BMP1 is enhanced when TWSG1 participates in the complex of Chordin and BMP (Xie and Fisher, 2005).

Mice deficient for TWSG1 have a number of developmental defects, including craniofacial malformations (Billington et al., 2011b; MacKenzie et al., 2009; Zakin and De Robertis, 2004), defects of the vertebrae, kidneys, thymus (Nosaka et al., 2003), and other organs that require branching morphogenesis such as salivary glands (Melnick et al., 2006). Given the role of TWSG1 in the development of branched organs, the goal of this study was to understand the role of TWSG1 during postnatal ductal maturation in the MG and to determine how perturbations in BMP signaling may affect that maturation.

Materials and Methods

Mice

Generation and genotyping of mice deficient for TWSG1 (C57BL/6 background) and heterozygous gene targeted Twsg1/Lac-Z knock-in mice have been previously reported (Gazzerro et al., 2006; Petryk et al., 2004). Use and care of the mice in this study were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Whole mount hematoxylin and X-Gal staining, elongation and branching analysis

Inguinal MGs (#4) were harvested from female mice in early puberty (Nelson et al., 1990) (day of vaginal opening, about six weeks of age). Some mice were allowed to recover and the contralateral MG was harvested at 10 weeks and stained as previously described (Li et al., 2002). MGs from 3 week old females were also collected. Mammary glands that were collected from 10 week animals were staged for metestrus by vaginal observation and vaginal smears. Briefly, whole glands were fixed in 4% paraformaldehyde (Polysciences, Inc. Warrington, PA) for 2 hours on ice, defatted with acetone and stained with hematoxylin (Fischer Scientific, Waltham, MA) cleared with xylenes and the gland flattened between two slides for image capture. For whole-mount lacZ staining, fat pads containing the mammary glands were isolated, fixed in ice-cold 2% formaldehyde, 0.2% gluteraldehyde, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 (NP40) in PBS for 5 min, washed with 2mM $MgCl_2$ in PBS, and stained overnight in X-gal solution [0.1 M phosphate pH 7.3, 2 mM $MgCl_2$, 0.01 % sodium deoxycholate, 0.02 % NP40, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$] supplemented with 1 mg X-Gal (Promega) on a rotating wheel at room temperature in the dark overnight. Following washing in PBS and refixing in 2% formaldehyde, 0.2% gluteraldehyde, the pads where photographed or dehydrated and processed for paraffin embedding and sectioning. The extent of fat pad colonization, secondary branching and TEB area were calculated as previously described (Khialeeva et al., 2011; McCaffrey and Macara, 2009; Richards et al., 2004). Briefly, the

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length and width of the ductal tree were measured as well as the length and width of the stromal fat pad. Areas were calculated for both, and a ductal tree: fat pad ratio was calculated. The extent of secondary branching was calculated by counting branch points within a $2 \times 3\text{-mm}^2$ area within 1mm of the lymph node and TEB area was calculated by using the freehand tool in ImageJ (Rasband, 1997– 2011) to trace the TEB, diameter was also calculated using the straight line tool.

Immunohistochemistry

Female mice at the onset of puberty were injected intraperitoneally (30 ng/g bodyweight) with bromo-2′-deoxy-uridine (BrdU) (Roche, Indianapolis, IN) and sacrificed 3 hours later. Inguinal MGs (#4) were harvested and fixed in 4% paraformaldehyde on ice for 2 hours, embedded in paraffin and sectioned at 5μM. Sections were deparaffinized in xylenes, rehydrated through a series of graded ethanols. For pSMAD1/5/8, GATA-3, ECAD, K8 and K14 staining, antigen retrieval was performed. Slides were placed in Antigen Unmasking Solution diluted to manufacturer's recommendation (Vector Laboratories, Burlington, CA) and microwaved for 20 minutes. Once cooled, slides were washed and then blocked in PBS containing 5% goat serum (Invitrogen, Carlsbad, CA) and 0.3% Triton X-100 (Fisher Scientific) for 60 minutes at room temperature. Sections were incubated with antipSMAD1/5/8 (Cell Signaling Technology Inc., Danvers, MA) 1:50, anti-GATA-3 (BD Pharmigen, San Diego, CA) 1:50, anti-E-Cadherin (Cell Signaling Technology Inc.) 1:100, anti-Keratin-14 (Covance, Princeton, NJ) 1:200, anti-Keratin-18 (TROMA-I) (Developmental Studies Hybridoma Bank, Iowa City, IA) 1:200 or anti-Progesterone receptor (Sigma) 1:50. Sections were developed either with Alexa Fluor (Invitrogen) or DyLight (AbCam, San Francisco, CA) species-appropriate fluorophore conjugated secondary antibodies. Fluorescence was visualized on a Zeiss 710 LSM Confocal
Microscope using an Argon12 laser (excitation 488 nm), a HeNe laser (excitation 543 nm) a HeNe laser (excitation 633). Images were taken under identical conditions and postacquisition manipulations were also identical.

Cell Culture

HC11 mouse mammary cells (Danielson et al., 1984) (a gift from Dr. Schwertfeger), derived from pregnant BALB/c mouse MG were cultured in RPMI 1640 medium (ATCC, Manassas, VA) supplemented with 10% fetal calf serum (ATCC), 53g/ml insulin (Sigma) and 10 ng/ml epidermal growth factor (Invitrogen). Cells were cultured in a humidified atmosphere of 5% CO2 in air at 37°C. Prior to BMP treatment cells were serum starved for 24 hours, and then treated with either BSA or recombinant BMP7 50ng/ml (R&D Systems).

Cell Proliferation and Apoptosis

BrdU incorporation was visualized using Bromo-2′-deoxy-uridine Labeling and Detection Kit II (Roche) following manufacturer's instruction and double stained with anti-K18 (Developmental Studies Hybridoma Bank) 1:200. Five TEBs per section (n=5) from 5 MGs were image captured. The number of BrdU positive cells and the total number of K18 positive cells were counted by 2 independent scorers who were blinded to genotype and the total number of BrdU positive cells are presented as a fraction of total nuclei. Using the same method, apoptotic cells were visualized with TUNEL staining (Gavrieli et al., 1992) using a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) following manufacturer's instructions. The total number of TUNEL positive nuclei within a TEB structure was expressed as a percentage of all nuclei within that structure.

cDNA generation, RT-PCR and qRT-PCR

Whole inguinal MGs (#4) were collected from WT and Twsg1$^{-/-}$ virgin female mice at the onset of puberty, lymph node removed, and MG lysed in TRIzol reagent (Invitrogen). Furthermore, inguinal MGs were collected and lysed in TRIzol from WT mice at 3 weeks, 10 weeks and early pregnancy

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(10.5 days past coitus). After extraction with TRIzol, RNA was isolated using RNeasy micro kit (Qiagen, Germantown, MD) following manufacturer's instructions. A similar procedure was followed to obtain total RNA from HC11 cells. Reverse transcription was carried out with the Thermoscript RT-PCR System (Invitrogen) priming with Oligo $(dT)_{20}$, followed by RT and qRT-PCR (Q-PCR, MX3000p, Agilent, LaJolla, CA) for BMP pathway components. Primers for BMP pathway components have been previously published (Sun et al., 2010). Our approach, given the expected reduced epithelium within the $Twsg1^{-/-}$ gland, was to first calculate a ratio between Gapdh and K18 and then use this ratio to normalize the signal of the gene of interest. This allowed us to look both at relative whole gland expression as well as relative epithelial expression.

Western blotting

Whole inguinal MGs were collected from virgin, female mice at the onset of puberty, lymph node removed and lysed in modified RIPA buffer (250ul of 140mM NaCl, 0.4 mM TrisHCl pH 8.0, 1% Glycerol, 1% NP40, 2% BSA with Complete Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche). SDS-PAGE was used to separate proteins and separated proteins were transferred to PVDF membrane. The membrane (Invitrogen) was blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) containing 0.1% v/v Tween 20. Membranes were incubated overnight at 4°C with anti-p-Smad1/5/8 (Cell Signaling
Technology Inc.) 1:250, anti-Total SMAD (Santa Cruz Biotechnology, Santa Cruz, CA) 1:100 and anti-GAPDH (ABCam, Cambridge, MA) 1:5000 antibodies and washed before incubation with species-appropriate fluorescent conjugated secondary antibodies for 1 h at room temperature. After washing to remove trace detergent, membranes were analyzed using an Odyssey Infrared Imaging System (LI-COR; Millennium Science, Surrey Hills, Australia) using the manufacturer's protocol. Relative pSMAD1/5/8 was calculated by first normalizing the signal intensity for total SMAD to GAPDH to control for loading. Then pSMAD1/5/8 signal was expressed as a percentage of the total SMAD pool. To detect GATA-3 membranes were incubated overnight at 4°C with anti-GATA-3 antibody at 1:50 dilution (AbCam, San Francisco, CA), probed with anti-mouse HRP-conjugated secondary antibody (Cell Signaling Technology Inc.), and visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Mammary gland transplantation

Mammary glands from 3 week old $Twsg1^{-/-}$ host females or WT host females were cleared of endogenous epithelium as described (DeOme et al., 1959). Donor mammary tissue was collected from adult wild type (WT) and $Twsg1^{-/-}$ mice and minced into small fragments. WT and $Twsg1^{-/-}$ mammary fragments were transplanted into the clear host fat pads, with each host (both WT and $Twsg1^{-/-}$) mouse receiving a WT transplant and a contralateral $Twsg1^{-/-}$ transplant. Host fat pads containing transplanted epithelium were removed and processed for whole-mount hematoxylin staining as described above. Approximately 80% of all transplants were able to colonize and elongate into the host fat pad.

Statistical analyses

Gene expression levels were normalized to K18/GAPDH and significance calculated using a Student's t-test with significance set at $p < 0.05$. pSMAD signal intensity was normalized to total SMAD which was first normalized to GAPDH. Significance was calculated using a Student's t-test with significance set at $p < 0.05$.

Results

TWSG1 and other BMP signaling pathway components are present in the mammary gland during postnatal development

To determine the presence and timing of TWSG1 expression during MG postnatal development, LacZ staining of mammary glands from heterozygous mice with LacZ inserted into the Twsg1 locus

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and RT-PCR for BMP pathway components was performed. Twsg1 was detected by LacZ staining in the myoepithelium and in a subset of body cells within the canalizing TEB (Fig. 1. A,B) at 6 weeks. In the mature gland, Twsg1 was expressed throughout the ductal tree and was primarily expressed in the myoepithelium (Fig. 1. C,D). Given that TWSG1 modulates BMP signaling, components of the BMP pathway were assessed by RT-PCR across developmentally relevant time points (Fig. 1E). mRNA for Bmp1, Bmp2, Bmp6, Bmp7, Bmpr1a, Bmpr2, and Smad4 were expressed across all time points. The Type1 receptor Bmpr1b was barely detectable. Bmp4 was detected at 10 weeks and at pregnancy. Among genes encoding BMP-binding proteins, Twsg1 mRNA was expressed at all time points while others were more temporally expressed. Chordin (Chrd) was undetectable at 6 weeks and barely detectable at other stages, while Chordin-like 1 (Chrdl1) was strongly expressed at each time point. Noggin (Nog) was expressed only during pregnancy while Chordin-like 2 (Chrdl2) was undetectable (Fig. S1). Cv2 mRNA was not detected during pregnancy, but was present at other time points.

TWSG1 is required for timely ductal elongation and fat pad colonization

MGs from WT and Twsg1$^{-/-}$ female mice were examined at 3, 6 and 10 weeks. The ductal trees were comparable at 3 weeks with the establishment of a rudimentary tree (Fig. 2A,D) although TEBs appeared atypical in the Twsg1$^{-/-}$ MG (Fig. 2G). At 3 weeks, TEBs were smaller than WT and in some cases it appeared as if TEBs were not established. At 6 weeks, the WT ducts had elongated well past the lymph node (LN) while the Twsg1$^{-/-}$ ducts showed little elongation. In most cases, the ducts did not reach or just reached the LN (Fig. 2B,E). The TEBs in the Twsg1$^{-/-}$ were larger than WT TEBs at this time point (Fig. 2H). At 10 weeks, the WT ducts had completely elongated, were well branched and the TEBs had reabsorbed. In Twsg1$^{-/-}$ ducts, TEBs were still present and the ductal tree was not as robustly branched (Fig. 2C,F). At 10 weeks, the WT TEBs had reabsorbed while TEBs were still found in the Twsg1$^{-/-}$ MG (Fig. 2I). A reduction in secondary branching in Twsg1$^{-/-}$

MGs at 10 weeks was also observed. The most striking difference between WT and Twsg1$^{-/-}$ ductal morphogenesis was found at 6 weeks and this stage was assessed further.

TWSG1 is required for timely elongation and fat pad colonization

MGs from Twsg1$^{-/-}$ female mice were examined on the first day of vaginal opening, approximately 6 weeks of age. MGs from WT animals contained ducts that extended past the lymph node and were robustly branched. In contrast, Twsg1$^{-/-}$ mice showed a significant decrease in fat pad colonization (Fig. 3A) with 38% of fat pad being occupied by the ductal network in Twsg1$^{-/-}$ MGs (n=5) compared to 64% in WT MGs (n=5, p=0.0032) at 6 weeks of age. In addition, secondary branching was significantly reduced in MGs from 6 week old virgin Twsg1$^{-/-}$ animals compared to WT (Fig. 3B) and TEBs were significantly larger in Twsg1$^{-/-}$ ducts (Fig. 3C).

Delay in elongation is intrinsic to the MG

To determine if the delay in ductal elongation and fat pad colonization was intrinsic to the MG rather than secondary to other factors, for example alterations in hormonal milieu, we performed transplantation experiments. We transplanted Twsg1$^{-/-}$ epithelium into the fat pads, cleared of endogenous epithelium, of 3 week old WT animals. The MGs were analyzed 3 weeks later and there was either a significant delay (n=2) or no measurable outgrowth (n=1) of Twsg1$^{-/-}$ MG tissue when compared to contralaterally transplanted WT tissue (Fig. 4A,B). Conversely, WT MG tissue transplanted into the cleared fat pad of Twsg1$^{-/-}$ had outgrowth that was similar to WT tissue

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transplanted into WT fat pad (data not shown). The TEBs in the $\text{Twsg1}^{-/-}$ transplanted tissue were similar in morphology to TEBs in 3 week $\text{Twsg1}^{-/-}$ MGs (Fig. 4C,D).

BMP signaling is reduced in the MG epithelium

To further investigate BMP signaling within the $\text{Twsg1}^{-/-}$ MG, a western blot was performed to detect pSMAD1/5/8. BMP signaling via pSMAD was significantly reduced in the $\text{Twsg1}^{-/-}$ MG (Fig. 5A). Co-localization studies demonstrated that in the WT MG pSMAD1/5/8 was localized to nuclei of cells within the TEB and pSMAD1/5/8 signal could not be detected in the $\text{Twsg1}^{-/-}$ TEB (Fig. 5B). We also observed that BMP downstream targets, Msx1, Msx2 and Gata-3, were downregulated in $\text{Twsg1}^{-/-}$ MG (Fig. 5C).

TWSG1 mediates lumen formation and luminal identity

Whole mount light microscopy of $\text{Twsg1}^{-/-}$ MGs showed a density of staining in the duct that was suggestive of a difference in cell density between WT and $\text{Twsg1}^{-/-}$ MGs. To investigate this further we sectioned MGs from 6 week old virgin females and stained with K18, K14 and Trichrome and assessed mature ducts. WT MGs at 6 weeks had mature ducts with a clear, well-defined lumen (Fig. 6A–C, G–I, M–N) with no cells visible within the lumen. Conversely, the $\text{Twsg1}^{-/-}$ MGs contained a mix of ducts containing cell islets (Fig. 6D–F), completely occluded ducts (Fig. 6J–L) as well as single cells shed into the lumen (Fig. 6Q–R) and clear lumens. Approximately 60% of the mature ducts in the $\text{Twsg1}^{-/-}$ MG had either complete or partial occlusion. When occluded ducts were stained for K14, the cells within the lumens contained an organized structure that consisted of a secondary K14 positive compartment surrounding a mass of K14 negative cells (Fig. 6D–F). This "duct within a duct" structure was only observed in $\text{Twsg1}^{-/-}$ MGs. WT, mature ducts consisted of a clearly defined luminal compartment that was K18 positive (Fig. 6G–I). Using a blood vessel landmark, the luminal compartments of $\text{Twsg1}^{-/-}$ ducts were further examined. In many cases, the duct remained occluded until out of the plane of sectioning and not all cells within the occlusion were K18 positive (arrows) (Fig. 6J–L). To look more closely at $\text{Twsg1}^{-/-}$ myoepithelial compartment, K14 stained sections were interrogated for the presence of basal body cells, which are K14 positive cells within the luminal compartment. In WT ducts, no basal body cells were observed (Fig. 6M–N), consistent with previous reports (Mailleux et al., 2007). Within $\text{Twsg1}^{-/-}$ MGs ~60% of the ducts observed contained K14 positive basal body cells. To further characterize the cells within the luminal compartment ducts were stained for smooth muscle actin (SMA) and a similar distribution of SMA positive cells were seen in the $\text{Twsg1}^{-/-}$ MG (data not shown). Additionally, we observed cell shedding in $\text{Twsg1}^{-/-}$ but not WT mature ducts. Usually these were single or small groups of cells within the lumens of mature ducts and these shed cells were K14 positive (Fig. 6Q,R).

Apoptotic defect may lead to accumulation of cells and poor lumen formation in *Twsg1* mutant ducts

The TEBs of MGs from $\text{Twsg1}^{-/-}$ animals are larger than WT controls suggesting hyperplasia. To assess this further we counted the number of cells within TEB structures (5 animals, 5 sections per animal) in WT and $\text{Twsg1}^{-/-}$ MGs. There was a significant increase in the number of K18 positive cells within the $\text{Twsg1}^{-/-}$ TEBs (Fig. 7A). To determine if this was due to an increase in proliferation or a decrease in apoptosis we stained for BrdU and performed a TUNEL assay. BrdU incorporation revealed no significant difference in proliferation between WT and $\text{Twsg1}^{-/-}$ TEBs (Fig. 7B,D). To determine if known regulators of proliferation are changed in our model, we assessed the expression of insulin-

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like growth factor 1, amphiregulin and estrogen receptor alpha by Q-PCR. There were no significant differences detected (data not shown). Furthermore, amphiregulin and progesterone receptor were visualized by IF and no differences in distribution were observed (data not shown). However, there was a marked decrease of nearly four-fold in apoptosis within the Twsg1$^{-/-}$ TEBs (Fig. 7C,D).

TWSG1 plays a role in restricting K14 positive cells to the basal compartment

Gata-3 is required for luminal identity in the MG and is a downstream target of BMP signaling in various tissues (Bonilla-Claudio et al., 2012). In WT TEBs (Fig. 8A–D) and mature ducts (Fig. 8I–L) almost all luminal cells expressed GATA-3. In contrast, Twsg1$^{-/-}$ MGs consisted of TEBs (Fig. 8E–H) and mature ducts (Fig. 8M–P) that were a mosaic of GATA-3 positive and GATA-3 negative cells. In Twsg1$^{-/-}$ MGs, single cells that were shed into the lumen were GATA-3 negative (Fig. 8P). To confirm that BMPs could induce Gata-3 expression in mammary epithelial cells, HC11 cells were treated with BMP7 50ng/ml and Gata-3 mRNA expression measured by Q-PCR. BMP7 induced Gata-3 expression over 2fold (p = 0.0004) (Fig. 9A). We also assessed Gata-3 expression and protein content and found that Gata-3 expression (Fig. 9B) as well as protein level (Fig. 9C) were reduced in Twsg1$^{-/-}$ MGs.

Discussion
TWSG1 as a positive regulator of BMP signaling during postnatal MG development

Studies in Drosophila melanogaster, Danio rerio, Xenopus, and Mus musculus have shown that TWSG1 is a highly conserved extracellular modulator of BMP signaling with important roles during embryonic development (Ross et al., 2001; Scott et al., 2001; Zusman and Wieschaus, 1985). Recent studies have demonstrated that TWSG1 continues to be an important BMP regulator in adult mammalian tissues, including bone homeostasis (Sotillo Rodriguez et al., 2009), regeneration following ischemic kidney injury (Larman et al., 2009), immune responses (Tsalavos et al., 2011), and, as this study shows, postnatal MG ductal maturation.

It is known that TWSG1 interacts with BMP2, BMP4, and BMP7 (Billington et al., 2011a; Blitz et al., 2003; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001), either directly or by forming complexes with CHRD (Larrain et al., 2001). While in Xenopus and Denio rario TWSG1 can act as a BMP antagonist as well as an agonist (Blitz et al., 2003; Ross et al., 2001; Xie and Fisher, 2005), the latter function has not been directly demonstrated in mammals. In this study, BMP signaling is reduced in the murine MG in the absence of TWSG1, suggesting pro-BMP activity in the postnatal MG. It is thought that the pro-BMP activity of TWSG1 results from its ability to facilitate the cleavage of CHRD by the MMP BMP1 in the extracellular space (Larrain et al., 2001). Chrd was not detected in the MG at 6 weeks, suggesting that CHRD-independent mechanisms may play a role (Xie and Fisher, 2005) A potential binding partner is CHRDL1, which is highly expressed in the developing MG and has been shown to bind BMPs and TWSG1. Intriguingly, in cells of the proximal tubule of the kidney, CHRDL1 amplifies BMP4 signaling in the presence of TWSG1 (Larman et al., 2009).

Reduced expression of the BMP targets Msx1, Msx2, Gata-3, and decreased levels of pSMAD1/5/8 all indicate a reduction of BMP signaling in the Twsg1$^{-/-}$ MG. Mutations in Msx1 and Msx2 result in impaired MG development. MGs of Msx2-deficient mice arrest at the mammary sprout stage while the epithelium of the Msx1/Msx2 double-deficient mice fails to form a bud with subsequent regression of the MG anlagen (Satokata et al., 2000).
Bmp2 and Bmp4 mutants are embryonic lethal (Lawson et al., 1999; Zhang and Bradley, 1996) and no studies have specifically addressed the effects of deletion of these genes in the postnatal MG.

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Thus, the Twsg1$^{-/-}$ mouse provides a model in which to evaluate the consequences of perturbated BMP signaling during postnatal ductal maturation.

TWSG1 is essential for postnatal ductal maturation in the murine MG

Postnatal MG development is orchestrated by numerous hormones, growth factors, cytokines, and transcription factors including MSX1, MSX2 and GATA-3, which regulate proliferation, apoptosis, and differentiation (McNally and Martin, 2011). Although BMPs are known to regulate proliferation through Msx1 (Zhang et al., 2002) and the expression of Msx1 was decreased in the Twsg1$^{-/-}$ MG, proliferation was not significantly altered suggesting that other pathways are involved in regulating proliferation in the elongating duct. In contrast, apoptosis was reduced in Twsg1$^{-/-}$ MG, indicating a role for BMPinduced apoptosis in lumen formation. Furthermore, Twsg1 is expressed in a subset of body cells within the TEB making it available for regulation of BMP signaling within the canalizing TEB. The classical mediator of the pro-apoptotic effects of BMPs is Msx2 (Graham et al., 1996). Indeed, reduced Msx2 expression was seen in the Twsg1$^{-/-}$ MG. The reduced apoptosis in the TEB could explain the observation that 60% of the mature ducts in the Twsg1$^{-/-}$ MG were occluded or had cell islets present in the lumen. It has been shown that disrupted apoptosis can be accompanied by lumen filling and cell shedding in the MG (Mailleux et al., 2007). However, it is also possible that cells shed into the lumen repopulate the lumen with a mass of cells that is later cleared by some unknown mechanism.

GATA-3 is directly induced by BMP signaling during facial skeletal development (BonillaClaudio et al., 2012), preplacodal ectoderm specification (Kwon et al., 2010), and hair follicle morphogenesis (Kobielak et al., 2003). In the MG, GATA-3 deficiency results in impaired placode formation, elongation defect as a result of the failure of TEBs to stably form, and a reduction in side branching (Asselin-Labat et al., 2007). Additionally, GATA-3 can induce apoptosis in a luminal tumor model (Kouros-Mehr et al., 2008) and upregulate pro-apoptotic genes such as caspase-14 (Asselin-Labat et al., 2011). In mice with MG specific GATA-3 deficiency lumens are irregular and cell shedding is observed (AsselinLabat et al., 2007). In the Twsg1$^{-/-}$ MG, loss of TWSG1 reduces BMP signaling perhaps by reducing BMP availability. As a result, not all cells reach the BMP signaling threshold required for Gata-3 induction and subsequent adoption of a luminal cell fate. The cells that fail to induce Gata-3 will remain in the luminal compartment or be shed into the lumen. It is possible that this mosaic of cell types in the advancing duct inhibits elongation but as cells are lost into the lumen cell:cell contacts are reestablished and elongation can again proceed in the absence of Twsg1$^{-/-}$.

We also observed a decrease in secondary branching in Twsg1$^{-/-}$ MG. As the ducts invade into the fat pad, secondary branching occurs in areas where there is low TGF3 expression (Pierce et al., 1993) and high Msx2 expression (Satoh et al., 2004). A recent study demonstrated that BMP2/4 can induce branching in the MG via Msx2 upregulation (Fleming et al.). When Twsg1 is deleted, some cells receive adequate BMP while others do not and in those regions with reduced BMP, Msx2 expression is also reduced and branching is not initiated, thus leading to an overall reduction in secondary branching in the Twsg1$^{-/-}$ MG.

Contribution of TWSG1 to maintaining basal vs luminal epithelial identity

The MG epithelium is composed of the basal myoepithelial cells and polarized luminal epithelial cells. It is thought that the different cell populations arise from multipotent progenitors, or mammary stem cells (Stingl et al., 1998). Interestingly, in Twsg1$^{-/-}$ MG, K14 positive cells are present within the luminal compartment among K18 positive cells. This suggests that BMP signaling plays a role in establishing and/or maintaining epithelial identity as opposed to myoepithelial, for example through GATA-3 (Metallo et al., 2008; Wilson and Hemmati-Brivanlou, 1995). In Twsg1$^{-/-}$ MGs, the K14

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positive cells within the luminal compartment may represent those cells whose BMP exposure was not sufficient to induce Gata-3 expression preventing progression towards a terminal luminal fate. In line with this, cells shed into the lumen of Twsg1$^{-/-}$ MGs are K14 positive whereas a previous study demonstrated that deleting Gata-3 after luminal specification did not reduce K18 expression showing that GATA-3 is not required for maintenance of K18, only its induction (Kouros-Mehr et al., 2006). This suggests that TWSG1 acts upstream of GATA-3 for regulating epithelial/myoepithelial cell fate.

In summary, this study sheds light on the role of the extracellular regulation of BMP signaling by TWSG1 during postnatal MG development. It shows that BMP signaling is reduced in MG leading to a decrease in Msx2 and Gata-3 expression. In the absence of TWSG1, delayed ductal elongation, impaired branching and lumen formation, incomplete myoepithelial restriction, and shedding into the lumen are observed. Understanding how TWSG1 regulates normal ductal maturation can provide insight into what role it may be playing during carcinogenesis, especially in light of the recent data suggesting tumor suppressor effects of BMPs (Loh et al., 2008; Owens et al., 2012; Ye et al., 2009). There is some evidence that the expression of Bmps, Msx2, and Twsg1 is altered in breast cancer (Finak et al., 2008; Malewski et al., 2005; Phippard et al., 1996), but the mechanisms for this differential regulation are unknown. Future studies will further elucidate the mechanism by which the lumens are eventually cleared in the absence of TWSG1. This may provide insight into how cells within the MG that escape targeted apoptosis at one point in development are identified and eventually cleared from the MG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- TWSG1 promotes BMP signaling in the developing mammary gland.

- TWSG1 null MGs have defects in ductal elongation, secondary branching and apoptosis.

- TWSG1 null MGs have occluded lumens and enlarged terminal end buds.

- TWSG1 null ducts fail to restrict myoepithelium to the basal layer.

- GATA-3, which is required for luminal identity, is reduced in TWSG1 null MGs.

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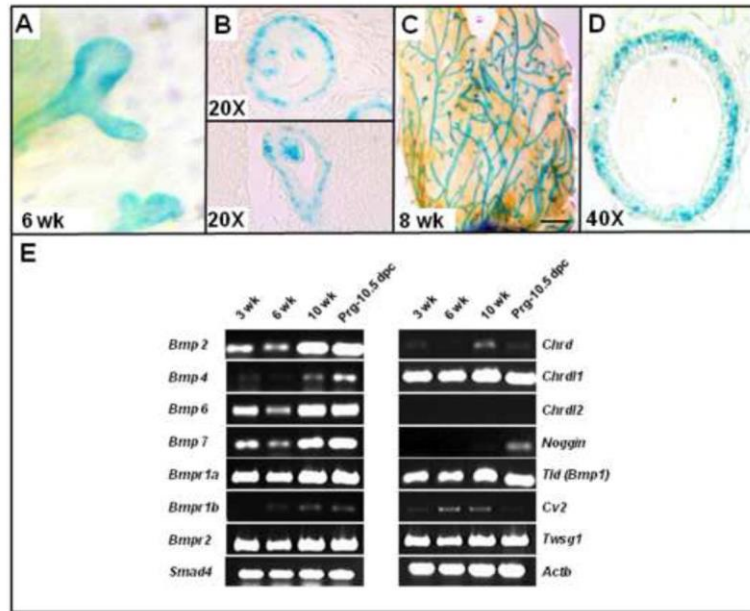
Fig. 1. *Twsg1* and BMP pathway components are expressed in the mammary gland (MG) at important postnatal developmental time points

(A–D) LacZ staining of whole mount (A,C) and sectioned (B,D) mammary glands isolated from heterozygous 6 and 8 week old virgin female carrying LacZ reporter gene in the Twsg1 locus. (A) Twsg1 is present in ducts and terminal end buds (TEBs) at 6 weeks (B) and in a subset of body cells in the canalizing TEB. (C) Twsg1 expression is present in the mature ducts (2 mm scale bar). (D) This expression is observed primarily in the myoepithelium at 8 weeks (E) RT-PCR showing temporal expression of BMP pathway members in MGs at different postnatal developmental time points. Actb, beta-actin, Chrd, chordin; Chrdl1, chordin-like 1; Chrdl2, chordin-like 2; Cv2, crossveinless 2.

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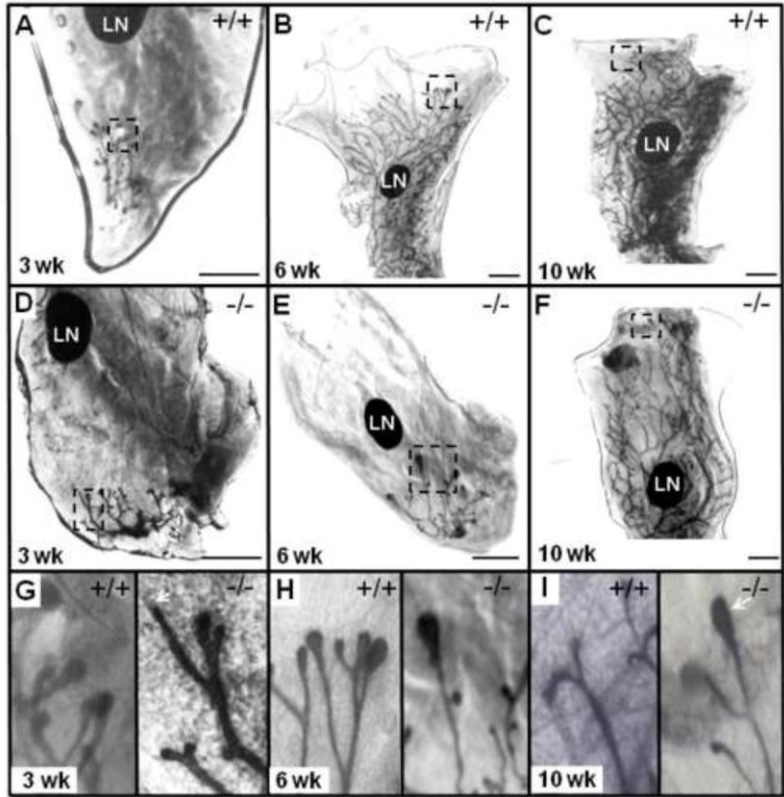
Fig. 2. Loss of TWSG1 results in a delay of ductal elongation

(**A, B, C**) Whole mount MG from 3 week, 6 week and 10 week old wild type (WT) virgin female mouse. (**D, E, F**) Whole mount MG from 3 week, 6 week and 10 week old Twsg1$^{-/-}$ virgin female mouse. (**G, H, I**) Distal ends of ductal tree from the MG of 3 week, 6 week and 10 week WT and Twsg1$^{-/-}$ virgin female mouse. Arrow indicates poorly formed TEB (2G) and TEBs still present at 10 weeks (2I) in the Twsg1$^{-/-}$ MG. Magnified distal ends are boxed. LN, lymph node. Scale bar 2 mm.
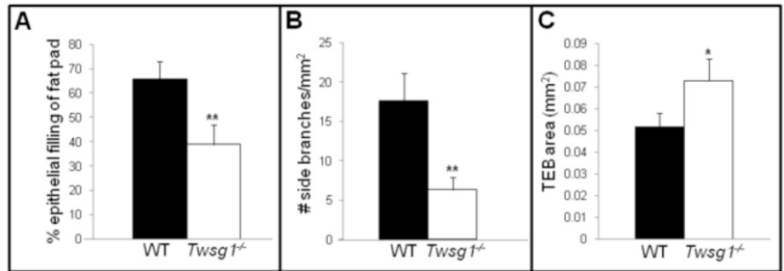
Fig. 3. Loss of TWSG1 results in impaired ductal elongation, reduced secondary branching and enlarged terminal end buds

(**A**) Percetage of fat pad colonization by the ductal tree in WT and Twsg1$^{-/-}$ virgin female mouse. (**B**) Side branching is significantly reduced in Twsg1$^{-/-}$ MG. (**C**) TEBs are significantly larger in Twsg1$^{-/-}$ MGs at 6 weeks. ** $p < 0.001$,

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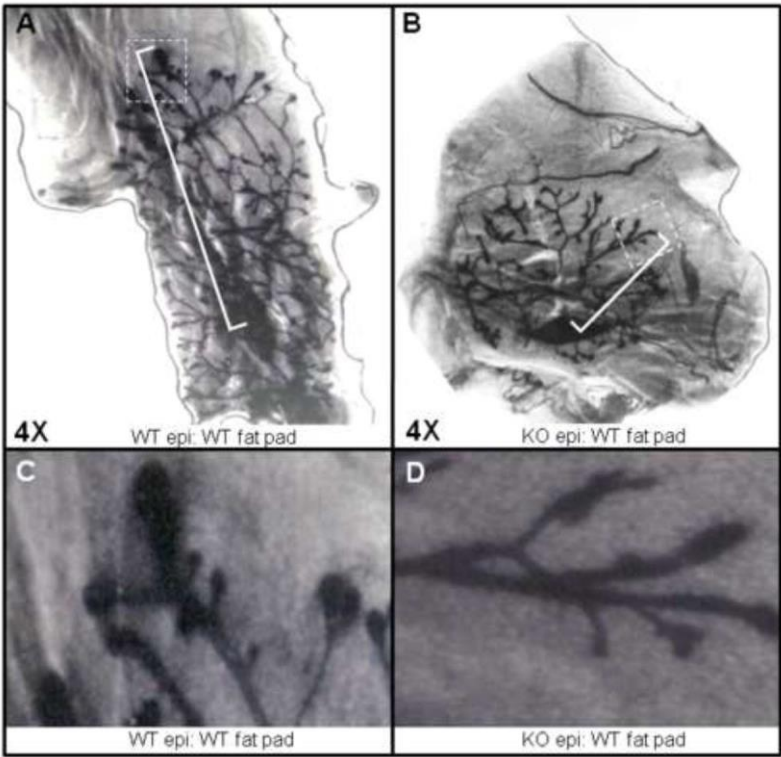
Fig. 4. Elongation defect is intrinsic to the MG

(**A**) WT epithelium transplanted into cleared fat pad of 3 week old WT mouse shows adequate elongation (n=3) as measured from center of transplanted epithelium to distal end of longest primary duct. Longest primary duct is bracketed and farthest distal end is boxed. (**B**) Twsg1$^{-/-}$ epithelium transplanted into cleared fat pad of 3 week old WT mouse shows impaired elongation (n=3) as measured from center of transplanted epithelium to distal end of longest primary duct. Longest primary duct is bracketed and farthest distal end is boxed.

(**C**) Magnified view of transplanted WT distal ends. (**D**) Magnified view of transplanted Twsg1$^{-/-}$ distal ends.
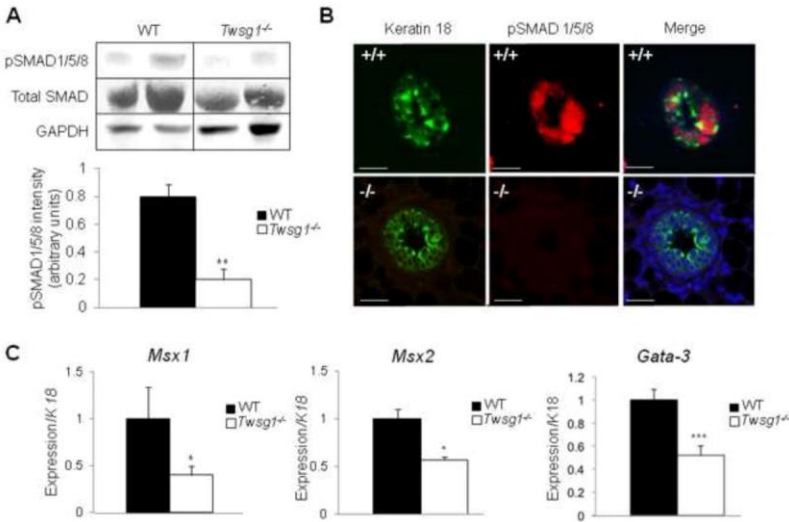
Fig. 5. BMP signaling is reduced in MGs from *Twsg1*$^{-/-}$ animals

(**A**) Western blot, showing two representative samples per group, detecting the levels of total SMAD, pSMAD 1/5/8 and GAPDH in MG lysates from WT and Twsg1$^{-/-}$ 6 week old virgins. Total SMAD was normalized to GAPDH and a ratio of SMAD to pSMAD1/5/8 was calculated; $p < 0.002$. (**B**) Immunofluorescence staining for epithelial marker keratin 18 (green) and pSMAD1/5/8 (red). pSMAD1/5/8 is absent in Twsg1$^{-/-}$ MGs (**C**) Q-PCR for BMP downstream targets, Msx1, Msx2 and Gata3, shows reduced expression in Twsg1$^{-/-}$ MGs. Scale bar 2 um.

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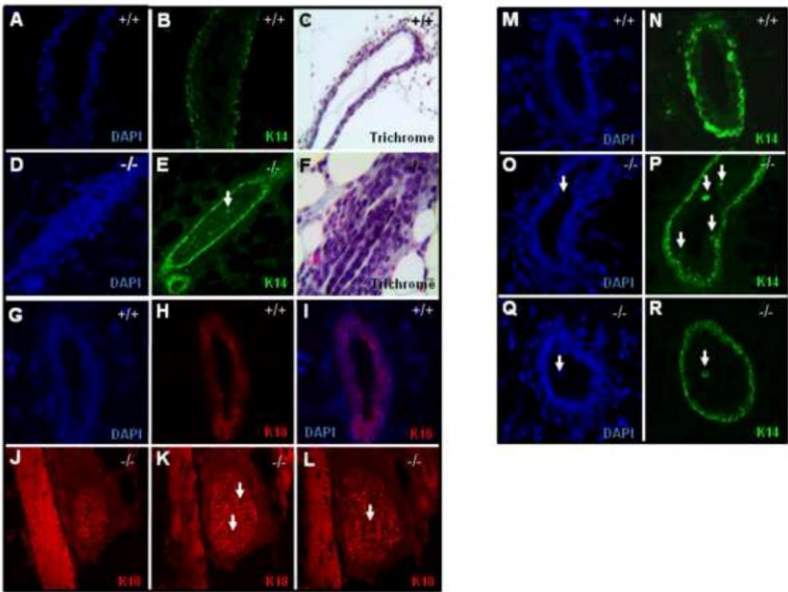
Fig. 6. *Twsg1*⁻/⁻ MGs have abnormal epithelial organization, occluded lumens and cell shedding at puberty

(A–C) WT MGs **(D–F)** Twsg1⁻/⁻ MGs stained with K14 have occluded lumens, which can be organized with a secondary myoepithelial compartment surrounding a mass of K14 negative cells. **(C,F)** Trichrome staining reveals cleared lumens of WT ducts and an organized cell mass within some lumens of the Twsg1⁻/⁻ maturing duct. **(G–I)** WT ducts with a well formed lumen. (J–L) Using a blood vessel to mark the location within the MG, serial sections of an occluded Twsg1⁻/⁻ duct were stained for K18. Not all the cells that occlude the lumen are K18 positive (arrows). **(M,N)** K14 stained WT mature ducts showing K14 positive cells in the basal compartment, but not the luminal compartment of the epithelium at puberty **(O,P)** K14 positive cells are found in the luminal compartment of Twsg1⁻/⁻ mature ducts (arrow). **(Q,R)** Cell shedding in Twsg1⁻/⁻ ducts. Cells shed into the lumen were preferentially K14 positive. All images were taken at 20X.


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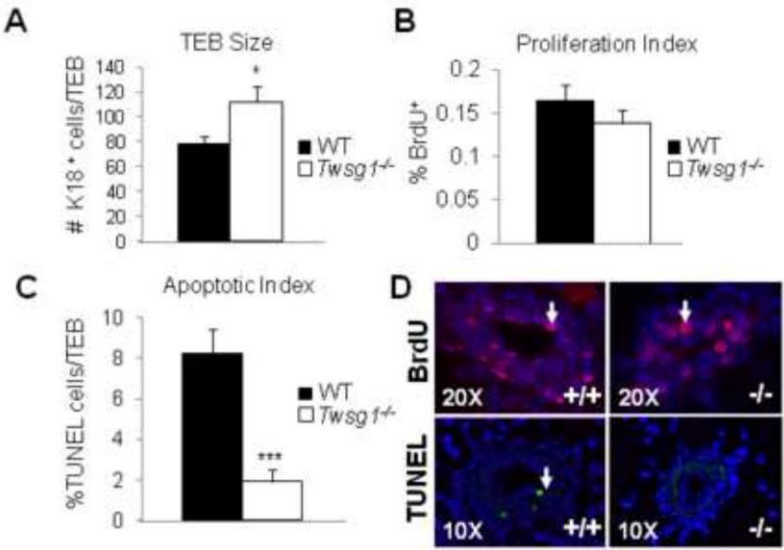
Fig. 7. *Twsg1*$^{-/-}$ TEBs show an increase in luminal epithelial (K18) cell number and a decrease in apoptosis

(A) K18 positive cells were counted within TEBs and compared between WT and Twsg1$^{-/-}$. A significant increase in cell number was observed in Twsg1$^{-/-}$ compared to WT. **(B)** BrdU positive cells were counted within the same compartment and no significant change in proliferation was observed. **(C)** TUNEL staining revealed a significant decrease in the number of apoptotic cells within the TEB of Twsg1$^{-/-}$ MGs. **(D)** Representative images for BrdU (pink), K18 (green) and TUNEL (green) staining. * $p<0.05$, *** $p<0.001$
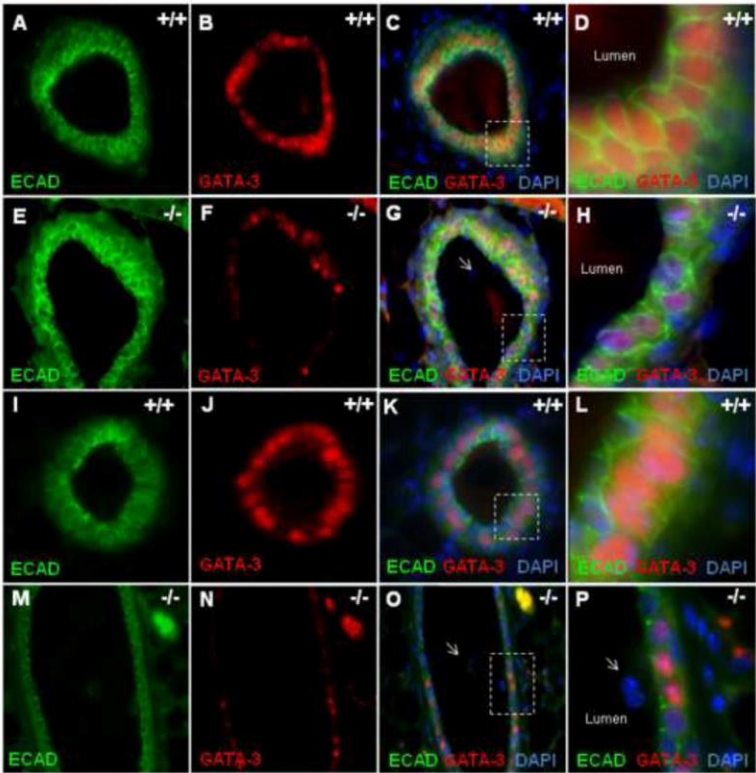
Fig. 8. GATA-3, required for luminal identity, is reduced in *Twsg1*$^{-/-}$ MGs GATA-3 expression is reduced in Twsg1$^{-/-}$ MGs. ECAD was used to mark luminal epithelial cells and most ECAD positive cells in the WT TEB express GATA-3 (A–D) and mature duct (I–L) while the Twsg1$^{-/-}$ TEB (E–H) and mature duct (I–L) consist of a mosaic of GATA-3 positive and GATA-3 negative luminal cells. Most cells shed into the lumen are GATA-3 negative (P). All images were taken at 40X. Boxes in C, G, K, and O correspond to the magnified images in D, H, L, and P, respectively.
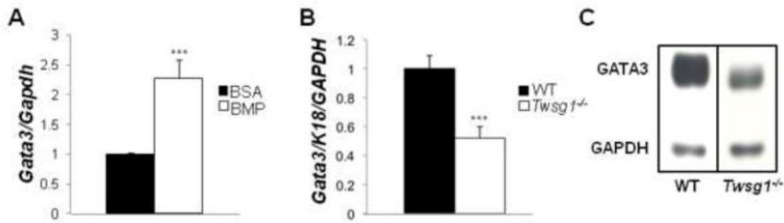
Fig. 9. GATA-3, required for luminal identity, is reduced in *Twsg1*$^{-/-}$ MGs

(**A**) Gata-3 expression is significantly upregulated in HC11 cells that have been treated with BMP7 (50ng/ml). (**B**) Gata-3 expression is significantly downregulated, in Twsg1$^{-/-}$ MGs from 6 week old virgin mice. (**C**) There is reduced GATA-3 protein in Twsg1$^{-/-}$ MGs (n=3) compared to WT (n=3).